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PRINCIPAL INVESTIGATOR: Xiankai Sun, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75390-9058

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Xiankai Sun, Ph.D.

E-Mail: Xiankai.Sun@UTSouthwestern.edu

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14. ABSTRACT The goal of this project is to develop dual modality imaging probes for the detection of prostate cancer by doping radioisotopes to iron oxide nanoparticles, so that the sensitivity and specificity of prostate cancer diagnosis could be significantly improved. In the three years, a facile approach has been developed to prepare gamma- or positron emitting nuclides incorporated SPIO nanoparticles (NUSPIONs) for dual modality imaging of prostate cancer, where a size-exclusion filtration method was introduced to expedite the preparation while maintaining the narrow size-distribution, high radiochemical purity, and consequently the uniform physical and chemical properties. The NUSPIONs were highly stable in vivo and showed high tumor-to-muscle ratio (12.2 ± 4.8 %ID/g at 24 h p.i.) in a prostate cancer xenograft model. Preliminary MR and autoradiography images with 177Lu-NUSPIONs in nude mice bearing PC-3 tumors clearly showed tumors implanted in the flanks, indicating the potential application of such nanoplatforms for the noninvasive dual modality imaging of prostate cancer.

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Introduction

The goal of this project was set to explore a new approach that will combine the advantages of MRI and PET for the diagnostic imaging and staging of prostate cancer. We propose to dope positron-emitting isotopes to superparamagnetic iron oxide nanoparticle to make nanosized dual MRI/PET probes for the detection of prostate cancer by multi-modality (anatomical MRI plus functional PET) molecular imaging approaches, so that the sensitivity and specificity of prostate cancer diagnosis could be significantly improved. To realize the goal, two objectives were specified for this project: **Objective I**. Preparation/characterization of $^{77/74}$As-doped iron oxide nanoparticles and construction of PSMA-targeted nano-conjugates; and **Objective II**. Evaluation of the PSMA-targeted nano-conjugates in prostate cancer xenograft mouse models via conventional biodistribution and small animal MRI/PET imaging methods.

Body

In the statement of work (SOW), the focus of our third year work was on part of **Objective I** and **Objective II.** Specifically,

Months 9 – 18 (partially accomplished):
Milestone: Establish protocols to construct PSMA-targeted nano-conjugates. Four such nano-conjugates are anticipated (two nano-conjugates with sizes of 25 nm and 35 nm per targeting molecule).
Months 12 – 30 (partially accomplished):
Milestone: Accomplish the in vitro/in vivo evaluations of the four PSMA-targeted nano-conjugates. At the end of this timeframe, we will be able to tell which targeting approach is better. We anticipate two nano-conjugates (one per targeting molecule) that can be used for small animal imaging studies.

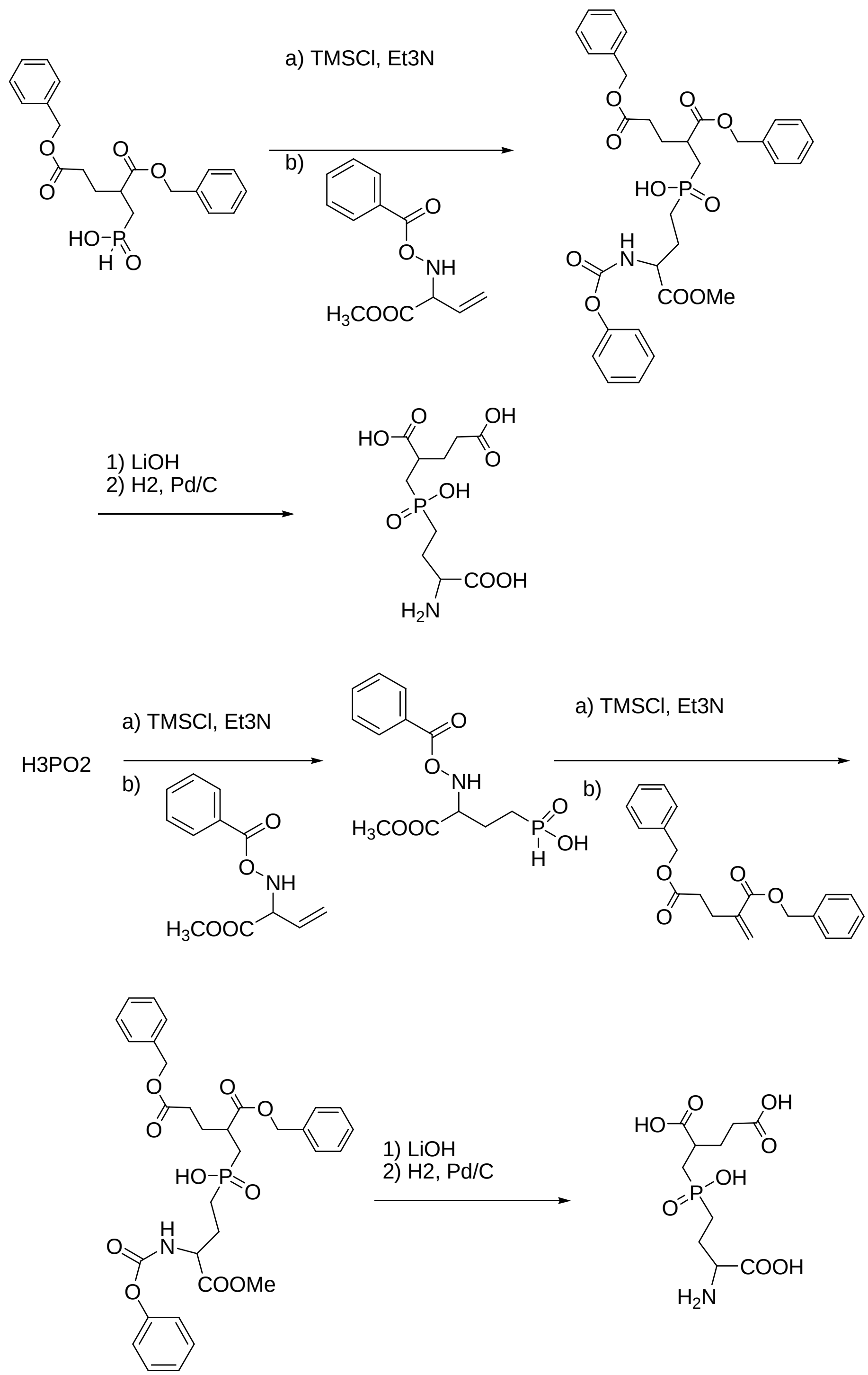
Months 24 – 36 (partially accomplished):
Milestone: Accomplish the small animal MRI and PET imaging evaluation of the two chosen nano-conjugates. We anticipate that the PSMA-targeted nano-conjugates can serve as dual-modality imaging probes, which will provide the desired higher sensitivity and specificity for PCa detection than either of the single-modality imaging approaches and the PSA test, by the combined analysis of MRI and PET images.

Research Progress in the Third Year:

In the second year, we established a standardized procedure to conjugate radioisotope-incorporated iron oxide nanoparticles (NP-1 and NP-2) with a prostate cancer targeting peptide, NH_2G-R11. However we have met two major problems, which impeded our progress:

Problem with the E6 antibody. In the 3rd year, we tested the E6 antibody three times for PSMA-targeted in vitro and in vivo evaluation. However it turned out that the antibody couldn't positively stain the C4-2 cells. Because Dr. Phil Thorp's laboratory (the antibody's provider) has terminated the E6 antibody related projects due to the lack of funding, we have to look for other PSMA-targeting antibody. Now we have identified two sources for the antibody supplies: abcam (http://www.abcam.com) and a research lab at Cornell University. We have done the C4-2 cell staining with an abcam's PSMA antibody (YPSMA-1). The result was convincingly positive. In addition to the antibody targeting approach, we have also resorted to small organic molecules. Currently we have nearly completed the synthesis of a reported PSMA-targeting molecule (GPI:

2- [((3-amino-3-carboxypropyl) (hydroxy) phosphinyl)-methyl] pentane-1, 5-dioic acid) as shown in Scheme 1.



Reference: Jackson et al. J Med Chem, 1996, 39, 619-622

Scheme 1. Synthesis of a PSMA-targeting molecule (GPI).

Arsenic-74 Supply Problem. We planned to import As-74 from University of Brussels in Belgium. However the cost has been doubled in the past years due to the weak dollar. So I was forced to seek a reliable source of As-74 in America. First I tried to persuade Washington University School of Medicine for the As-74 production. After a few months of communication, we couldn't overcome the radiation safety regulation problems in the state of Missouri, so I turned to the Isotope Programs of DOE. With the help of Prof. Michael Welch (my postdoc mentor), the program manager, Dr. Wolfgang Runde (runde@lanl.gov), has agreed to have the Los Alamos National Laboratory produce As-74 for us. Now we are about to get the production started.

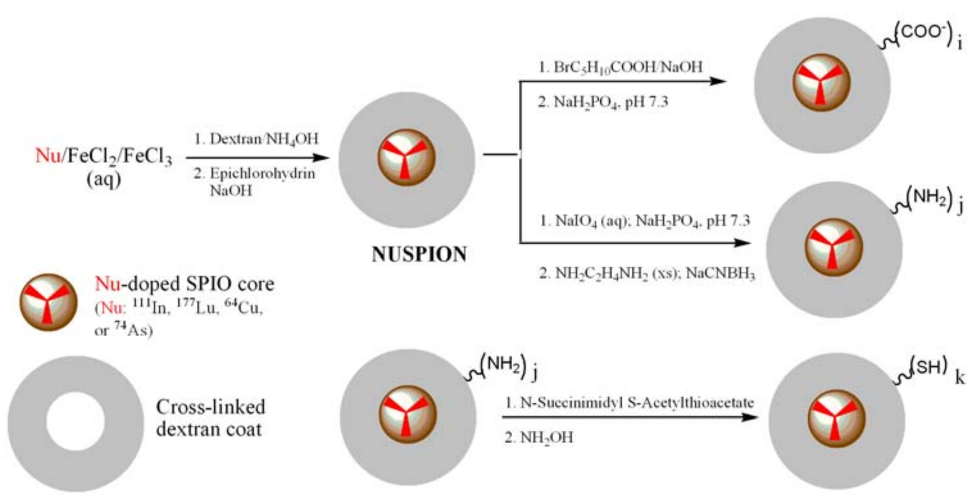
In spite of the above obstacles, we have one manuscript that has been accepted for publication on the Journal of Biomedical Nanotechnology and one manuscript to be submitted (within a couple months). The manuscripts describe the preparation and evaluation of two types of radioisotope-incorporated iron oxide nanoparticles for dual modality imaging: Dextran-coated and Gold-coated nanoplatforms. Encouragingly we have achieved the dual-modality imaging of prostate tumors simultaneously using MRI and autoradiography in the 3rd year.

Key Research Accomplishments

In summary, in three years we have accomplished the following aims:

1. Standard Operation Procedures (SOP) for the Synthesis of multifunctional nanoscaffolds

The standard operation procedures (SOP) for the synthesis of NUSPIONs (NUSPION: gamma- or positron-emitting nuclides incorporated dextran-coated SPIO nanoparticles) and the following surface functionalization have been established as shown in Scheme 1. In the procedures, we adopted a size-exclusion centrifugation technique to expedite the separation and purification of nanoparticles, which typically takes less than 2 hours rather than over 10 hours if using dialysis tubes or lengthy column separation as in the preparation of SPIO. In addition, most of the reaction conditions are controlled by digital devices. To date we have run more than twenty times of such preparation and been able to create a panel of NUSPIONs with carboxylate, amino, or thiol groups on the surface for further conjugations if required by different functional molecules. The results (nanoparticle size and size distribution, radioisotope incorporation rate, and derivatization efficiency) have been reliable and reproducible. For the four isotopes, the incorporation rates are always above 70% in our current reaction scales.



Scheme 1. Preparation of NUSPIONs and thereafter surface derivatization to form carboxylate, amino, or thiol groups for further multi-presentation of functional molecules.

2. Characterization of NUSPIONs

Size and size distribution: Two typical NUSPIONs are routinely prepared in our laboratory: NUSPION-1 and NUSPION-2. The hydrodynamic sizes (radii) of NUSPION-1 and NUSPION-2 in aqueous solutions as measured by dynamic light scattering (DLS, Wyatt's DynaPro) are 11.8 ± 1.5 nm and 25.2 ± 2.1 nm (> 20 trials), respectively (Figure 2); their SPIO core sizes determined by TEM ((FEI Tecnai G2 Spirit BioTWIN Microscope) are 6.2 ± 1.2 nm and 13.0 ± 3.2, respectively. As shown in Figure 1, both NUSPION-1 and NUSPION-2 are virtually mono-dispersed. NUSPIONs are also be characterized by atomic force microscopy (AFM). Shown in Figure 2, the purification of NUSPIONs by our own developed size-exclusion centrifugation method is rather efficient.

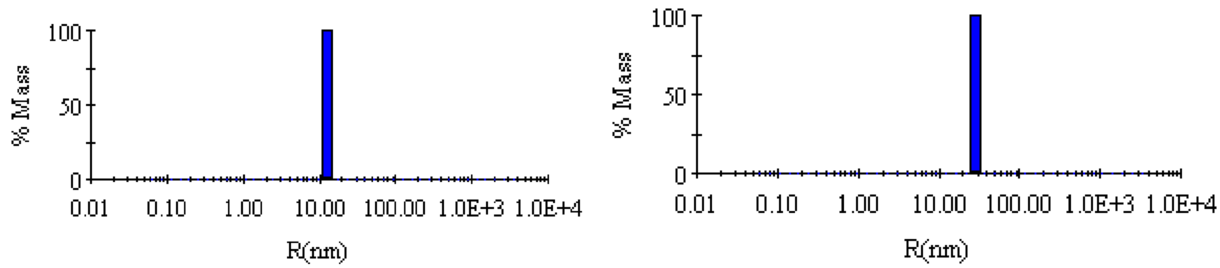


Figure 1. DLS results of ^{177}Lu-doped NUSPION-1 (left: 12.1 nm) and NUSPION-2 (right: 25.2 nm).

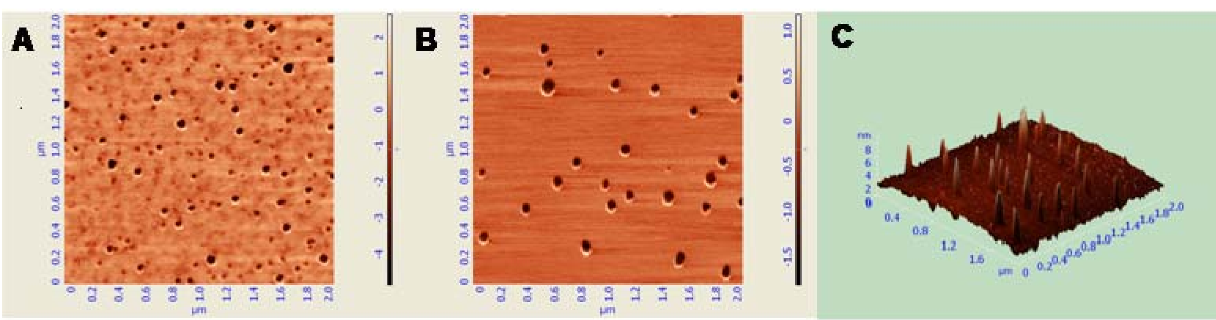


Figure 2. Representative AFM images of NUSPIONs on MICA substrate before (A) and after (B) separation/purification, and (C) the corresponding 3D image of (B).

3. Quality Assurance by HPLC: The integrity of NUSPIONs is critically important for their applications. We have developed a sensitive and reliable method by using an HPLC system equipped with three different detectors: a Wyatt Mini DAWN light scattering detector for the nanoparticles, a Waters UV2996 PDA for a wide range of UV detection of functional molecules anchored on the surface of NUSPIONs as well as the particles themselves; and an HPLC radiodetector for the radioisotopes. Shown in Figure 3 is a typical stack display of the HPLC spectra acquired from the three detectors. By the three HPLC readouts, we can convincingly and accurately determine the integrity of NUSPION-based multifunctional probes (all functional components are present in the same nanoscaffold) and their in vitro and in vivo stability. In addition, this HPLC method can be used to monitor the chemical reactions in Schemes 1&2 and the separation/purification procedures. It is noteworthy that the UV peak intensity (area) change

at a certain wavelength before and after a molecule conjugation can be used to quantify the number of the molecule that have been linked to the NUSPION surface.

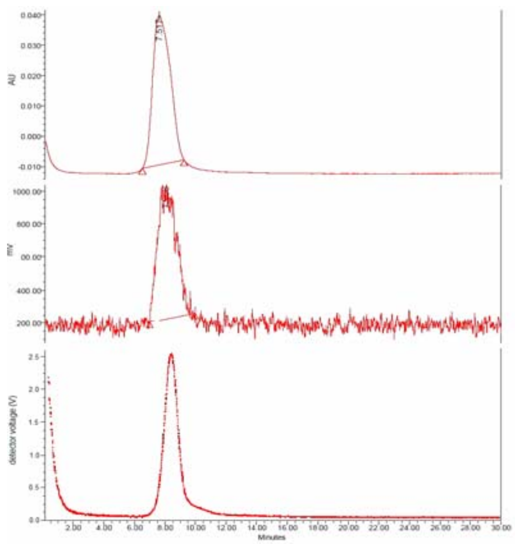


Figure 3. Stack display of three HPLC readouts of a ^{177}Lu-doped NUSPION (top: UV by PDA; middle: radioactivity; bottom: light scattering).

<u>4. In vivo distribution of NUSPIONs</u>

In normal balb/c mice. The in vivo evaluation of NUSPIONs was firstly performed in normal balb/c mice using two ^{177}Lu-doped NUSPION-1 and NUSPION-2. The biodistribution data are presented in Figure 4. NUSPION-1 showed significant higher blood uptake and lower accumulation in liver and spleen than NUSPION-2 at both 1 h and 4 h post-injection (p.i.) ($p < 0.005$). The higher uptake and longer retention of NUSPION-1 in blood is a desired feature for a nanoscaffold to be used for imaging and/or drug delivery by either passive or active tumor targeting mechanism. Interestingly, NUSPION-1 might be able to be cleared by renal filtration because it showed significantly higher uptake in kidney than its larger counterpart (NUSPION-2) ($p < 0.005$) with 48 h p.i. Because NUSPION-1 exhibited a more optimal tissue distribution profile than NUSPION-2, it was further evaluated in a tumor-bearing animal model.

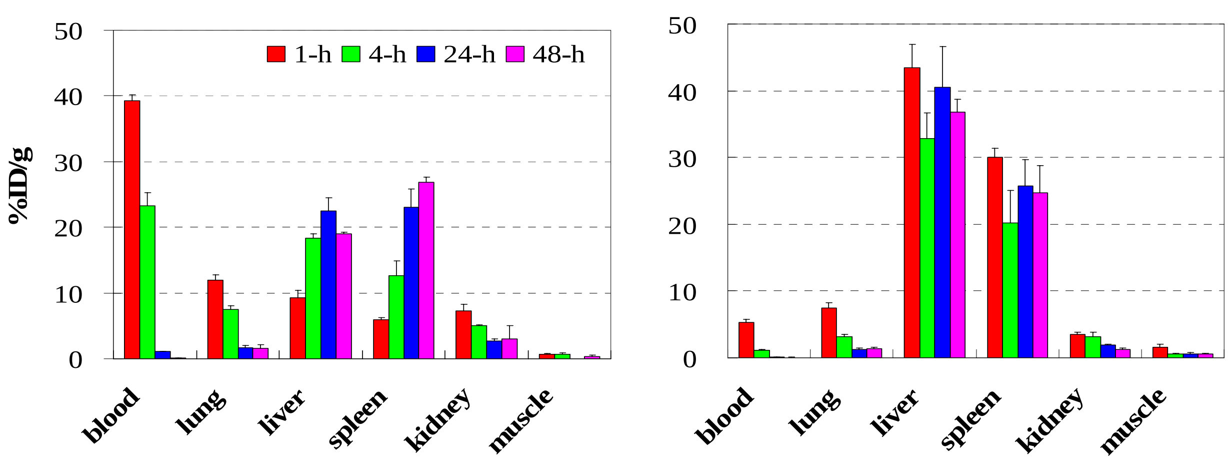


Figure 5. Biodistribution of ^{177}Lu-doped NUSPION-1 (left) and NUSPION-2 in normal balb/c mice (n = 4).

In PC-3 tumor bearing mice. The tissue distribution profile of ^{177}Lu-doped NUSPION-1 (24 h and 48 h p.i.; n = 4) in this nude mouse model was similar to that in normal mice (Figure 5, left). Indeed NUSPION-1 showed significant accumulation in the tumors (each mouse bearing two PC-3 xenografts (~ 8 mm) in both back flanks). Summarized in Table 2, the mean ratios of tumor to muscles (T/M) and tumor to blood (T/B) are well above 10 at both time points. The passive

Table 2. Tumor uptake of NUSPION-1 in PC-3 tumor bearing mice

	Tumor uptake (%ID/g)	T/M	T/B
24-h	2.2 ± 0.8	12.1 ± 3.9	12.9 ± 3.9
48-h	1.2 ± 0.4	13.3 ± 5.9	31.6 ± 17.3

tumor targeting was likely resulted from the enhanced permeability and retention effect due to the leaky vasculature and poor lymphatic drainage in solid tumors. It is reasonable to assume that the targeting property of NUSPION-1 would be improved once tumor targeting molecules are used.

4. Dual-modality imaging evaluation of NUSPIONs

The same tumor bearing animal model was used for the proof-of-concept dual modality imaging studies, in which both MRI and nuclear imaging were applied to the same subject injected with the dual modality imaging agent (^{177}Lu-doped NUSPIONs). Because the incorporated radioisotope amount can be conveniently controlled in the preparation outlined in Scheme 1, we were able to inject the right amount of dose to enable the dual modality imaging studies. Specifically an injection dose of ^{177}Lu-doped NUSPION-1 was prepared to contain 0.1 mmol/kg of iron, which is a typical SPIO dose for MRI scans, and ~ 100 μCi of ^{177}Lu-activity, a typical dose for mouse SPECT imaging.

Before injection, the tumor bearing mice (n = 4) were scanned by a 4.7 T magnet to collect the imaging baseline. After injection, the mice were imaged at 1 h, 3 h, and 24 h p.i. under anesthesia. Multi-slice T_2 maps were obtained at these time points using a spin echo sequence. ROI analysis was performed on a slice-by-slice basis using homebuilt Matlab routines and the T_2 values from all voxels of each tumor at each time point were pooled for statistical comparison using a t-test using Origin® program (OriginLab Corp., Northhampton, MA). Although two tumors were implanted on each mouse, only one tumor/mouse which showed minimal effect of motion artifacts, was used for analysis. After the MR imaging, the mice were imaged by a PerkinElmer autoradiography system (Cyclone) at 26 h and 72 h p.i. The exposure time was 60 s or 90 s.

As shown in Figure 5 (left), compared to the value before injection, a small but statistically significant decrease of the mean T_2 value was observed at both 1 h and 3 h p.i. ($p < 0.001$). On the autoradiography images, the tumors in both flanks are clearly visualized at 26 h and 72 p.i. Evidently dual modality non-invasive imaging of PC-3 tumors has been achieved by a single injection of our developed dual modality imaging probe.

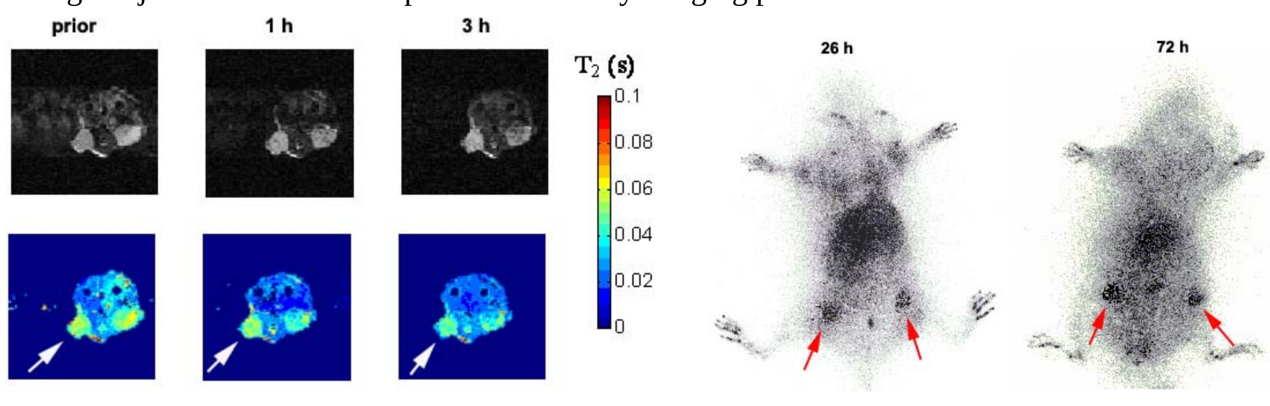


Figure 6. Left: MR imaging of a PC-3 tumor bearing mouse before and after injected with NUSPION at a dose of 0.1mmole/kg (Fe); Right: Autoradiography images of the same mouse at 26 h and 72 h p.i. The white or red arrows indicate tumors.

Reportable Outcomes

One manuscript has been accepted by the Journal of Biomedical Nanotechnology

One manuscript in preparation is to be submitted next month.

In the 12-month extension, we will (1) prepare As-74 incorporated iron oxide nanoparticles for the construction of PSMA-targeted nanoconjugates; (2) synthesize and evaluate two PSMA-targeted nanoconjugates using one PSMA antibody and the PSMA-targeting small organic molecule for dual modality MRI/PET imaging of prostate cancer to accomplish the goals (milestones) set in this project.

Conclusions

A facile approach has been developed to prepare gamma- or positron emitting nuclides incorporated SPIO nanoparticles (NUSPIONs) for dual modality imaging of prostate cancer, where a size-exclusion filtration method was introduced to expedite the preparation while maintaining the narrow size-distribution, high radiochemical purity, and consequently the uniform physical and chemical properties. The NUSPIONs were highly stable in vivo and showed high tumor-to-muscle ratio (12.2 ± 4.8 %ID/g at 24 h p.i.) in a prostate cancer xenograft model. Preliminary MR and autoradiography images with ^{177}Lu-NUSPIONs in nude mice bearing PC-3 tumors clearly showed tumors implanted in the flanks, indicating the potential application of such nanoplatforms for the noninvasive dual modality imaging of prostate cancer.